ISSN (print) 0093-4666 © 2014. Mycotaxon, Ltd. ISSN (online) 2154-8889

MYCOTAXON

http://dx.doi.org/10.5248/128.93

Volume 128, pp. 93–104 *April–June 2014*

Muscodor strobelii, a new endophytic species from South India

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Abstract — *Muscodor strobelii* is described as a new endophytic species existing in stems of *Cinnamomum zeylanicum* from South India based on morphological and molecular evidences along with volatile organic properties.

Key words — antimicrobial activity, sterile ascomycete, cinnamon, ITS- rDNA

Introduction

Plants hold an enormous diversity of fungal and bacterial endophytes within leaf and stem tissues, without any obvious indication of their presence (Bacon & White 2000, Schulze & Boyle 2005, Strobel 2006, Murali et al. 2007, Hyde & Soytong 2008, Rodriguez et al. 2009, Peay et al. 2010). Endophytes have been recognized as a promising resource of bioactive compounds and phytochemicals, which find applications in medicine, industry, and agriculture (Strobel & Daisy 2003, Mitchell et al. 2008, Tejesvi et al. 2009, Aly et al. 2010, Gutierrez et al. 2012).

Eight sterile endophytic fungi emanating distinct musky odours due to presence of volatile organic compounds (VOCs) have been described as novel fungi: *Muscodor albus* from *Cinnamomum* in Honduras (Worapong et al. 2001); *M. cinnamomi* from *Cinnamomum* in Thailand (Suwannarach et al. 2010); *M. crispans* from *Ananas* in Bolivia (Mitchell et al. 2008); *M. fengyangensis* from *Actinidia* and *Pseudotaxus* in China (Zhang et al. 2010); *M. roseus* from *Grevillea* in Australia (Worapong et al. 2002); *M. sutura* from *Prestonia* in Colombia (Kudalkar et al. 2012); *M. vitigenus* from *Paullinia* in Peru (Daisy et al. 2002); and *M. yucatanensis* from *Bursera* in Mexico (Gonzalez et al. 2009).

Apart from morphological and molecular differences, gas chromatography and mass spectroscopy (GC/MS) of VOCs have also been used as a tool to demarcate different species of *Muscodor* (Strobel et al. 2001, Kudalkar et al.

2012). *Muscodor* species exhibit biological control properties due to their VOC admixtures that exhibit antibiotic properties and induce lethal effects on insects and nematodes.

In this study, we recovered an endophytic fungus (#6610) from the stem tissue of a cinnamon tree (*Cinnamomum zeylanicum*) growing in Biligiri Rangaswamy Temple (BRT) wildlife sanctuary, India. Its cultural, chemical, and molecular characters are distinct from previously described *Muscodor* species, and so we name it here as *Muscodor strobelii*.

Materials & methods

Fungal isolation

Healthy and mature twigs were collected from *Cinnamomum zeylanicum* growing in BRT Wildlife Sanctuary, Karnataka, India, during July 2009. The plant parts were placed in sterile zip pouches and stored at 4 °C until further use. Fungi were selectively isolated using VOC stress assay as described by Ezra et al. (2004) using the *Muscodor albus* CZ620 type strain (gifted by Professor Gary A. Strobel, Montana State University, USA). The twigs were cut into 1–2 cm pieces under aseptic conditions, surface sterilized (with 1% sodium hypochlorite for 5 min, 70% ethanol for 1 min, and 30% ethanol for 30 s), and air dried under a laminar hood. The sterilized plant samples were cut into 5–10 mm segments, placed into a potato dextrose agar (PDA) plate containing a 7-day old culture of *M. albus* CZ620, and sealed with parafilm. The plates were incubated at 24 ± 2 °C for a fortnight with a 12 h photoperiod and periodically observed for mycelial germination. The fungi growing out of the plant tissue were aseptically picked from the tip and transferred to fresh PDA plates so as to obtain pure isolates. VOCs produced by *M. albus* CZ620 would permit growth of only volatile-tolerant fungi, thus enhancing the probability of finding a new *Muscodor* isolate or strain. The pure isolates thus obtained were maintained on PDA slants. Combinations of various media [Richard's agar (RA), corn meal agar (CMA), banana leaf agar (BLA), malt extract agar (MEA)] and growth conditions were tested to stimulate spore production (Guo et al. 1998). The morphological characters (e.g., colony color, texture, growth pattern, pigment formation, VOCs) and microscopic structures (e.g., hyphal characteristics, cellular bodies) were observed (Mitchell et al. 2008). The holotype was deposited as a metabolically inactive culture (liquid nitrogen) at National Fungal Culture Collection of India, Agharkar Research Institute, Pune (NFCCI).

Scanning electron microscopy

The morphology of isolate #6610 was observed under the scanning electron microscope following Ezra et al. (2004). The samples were placed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.2) overnight at 4 °C. The next day these were washed twice with 0.1 M phosphate buffer for 10 minutes each, and then washed for 10 minutes each in 30%, 50%, 60%, 70%, 80%, 90%, 95% and 100% acetone. The samples were then dehydrated to critical point using liquid CO_2. Gold palladium coating of dehydrated fungal samples was done using a sputter coater and images were recorded in high vacuum mode using Zeiss Evo40 scanning electron microscope (Ezra et al. 2004, Kudalkar et al. 2012).

Fungal DNA isolation and amplification

Isolate #6610 was grown on PDA in a 90 mm petri dish for 7 days, after which the mycelium was harvested, freeze dried in liquid nitrogen, and ground into a fine powder with pestle and mortar. About 10 mg of powdered mycelium was transferred to 2 ml microfuge tube and genomic DNA extracted using Wizard® Genomic DNA purification kit (Promega, USA) as per manufacturer's protocol.

The internal transcribed spacer (ITS) regions 1 and 2 and 5.8S rDNA regions were amplified using *Muscodor* specific primers *M. albus* F (5′-GGGAGGCTACCCTATAGGGGATAC-3′) and *M. albus* R (5′-CAGGGGCCGGAACCAC TACAGAGG-3′) (Ezra et al. 2010). The 25 µl reaction mixture for ITS amplification comprised 1 µl of extracted fungal DNA, 10 µM of each primer, 2.5 mM of dNTP, 25 mM $MgCl_2$, and 1.5 U of Taq DNA polymerase in 10 X Taq buffer (Bangalore GeNei). Thermal cycling conditions for *Muscodor* specific primers was initial denaturation at 96 °C for 5 min followed by 35 cycles of 95 °C for 45 s, 60 °C for 45 s, 72 °C for 45 s, followed by final extension at 72 °C for 5 min. The ITS amplicons were examined using a 1.5% agarose gel under UV light in Bio-Rad Gel documentation System using Quantity-1-D analysis software. The PCR products were purified with Wizard® SV gel and PCR clean up system (Promega, USA) following manufacturer's protocol. The purified products were directly sequenced (Chromus Biotech, Bangalore) using PCR primers mentioned above.

Sequence assembly, alignment and phylogenetic analysis

Sequences were assembled using Sequencher ver. 5 (www.genecodes.com) and compared to GenBank sequences using the BLAST software on the NCBI website. The ITS sequences were aligned with selected sequences of reference taxa obtained from BLAST using the Clustal W. Any duplicate sequence types were removed from the data set prior to phylogenetic analyses. After multiple alignments, phylogenetic analysis was conducted in MEGA 5.0.5. A phylogeny comparing *Muscodor* species #6610 with related taxa was generated by the Neighbour-Joining method (Saitou & Nei 1987). The evolutionary distances (base substitutions per site) were computed using the Kimura 2-parameter method (Kimura 1980). The rate variation among sites was modeled with a gamma distribution (shape parameter = 5). Alignment gaps were designated as missing data. Clade stability was assessed by bootstrap analysis with 1000 bootstrap replicates. The phylogenetic tree (Fig. 8) was drawn and edited in MEGA 5.0.5.

Qualitative analysis of volatiles

The volatile organic gas mixture emanated by the 10-day old isolate #6610 culture was entrapped using a solid phase micro-extraction (SPME) syringe with a stable flex fibre of 50/30 di-vinylbenzene/carboxen on polydimethylsiloxane (Supelco, Sigma Aldrich) following Ezra & Strobel (2004). The fibre was exposed to the air space above the fungus for 45 min by placing the SPME syringe after drilling a small hole with the help of sterile needle. The exposed fibre was injected for 30 s in the Shimadzu QP 2010 + gas chromatograph with thermal desorption system TD 20. A RTX column (diphenyl 95%, dimethyl polysiloxane 5%) with 30 m × 0.25 mm ID and 0.25 mm DF was used to separate the fungal volatiles. The column was programmed at 100 °C for 2 minutes before the temperature was increased to 250 °C for 2 minutes and finally to 300 °C

for 13 minutes. The carrier gas was helium and the initial column head pressure was 94.4 KPa. Data acquisition and processing was done on GCMS solution software. The compounds obtained after GC/MS analysis was then subtracted from the control plate consisting only PDA medium. The obtained compounds were then tentatively identified based on their high quality matching with database of National Institute of Standard and Technology (NIST) compounds (NIST05) and compared with all reported species of *Muscodor* (Ezra et al. 2004, Kudalkar et al. 2012).

Bioassay of VOCs produced by *Muscodor strobelii*

Antimicrobial spectrum of volatiles produced by *Muscodor strobelii* (#6610) was tested by uncomplicated bioassay using 90 mm four-quadrant petri dishes (Ezra et.al. 2004). A divided petri dish was used to prevent movement of any diffusible inhibitory compound from *M. strobelii* to the test panel microorganisms through the medium. One quadrant of the petri plate was inoculated with the mycelial plug of an actively growing *M. strobelii* culture and the plates were sealed with parafilm and incubated for 5 days at 24 ± 2 °C for production of the VOCs. Subsequently test bacteria and yeast were streaked in rest of the quadrants. While testing filamentous pathogenic fungi 3 mm mycelial plugs were inoculated. Correspondingly the control plates comprised only inoculated test bacteria or fungi and were devoid of isolate #6610 allowing it to grow normally. The antimicrobial action of VOC was determined by monitoring the growth of the test organism in the control and test plates. To check the VOC inhibitory or killing effect after 3 days exposure, the exposed culture plugs were placed on fresh solid media (PDA, YEPD, MHA) to assess their viability (Mitchell et al. 2010).

Taxonomy

Muscodor strobelii Meshram, S. Saxena & N. Kapoor, **sp. nov.** Figs 1–7

MycoBank MB802337

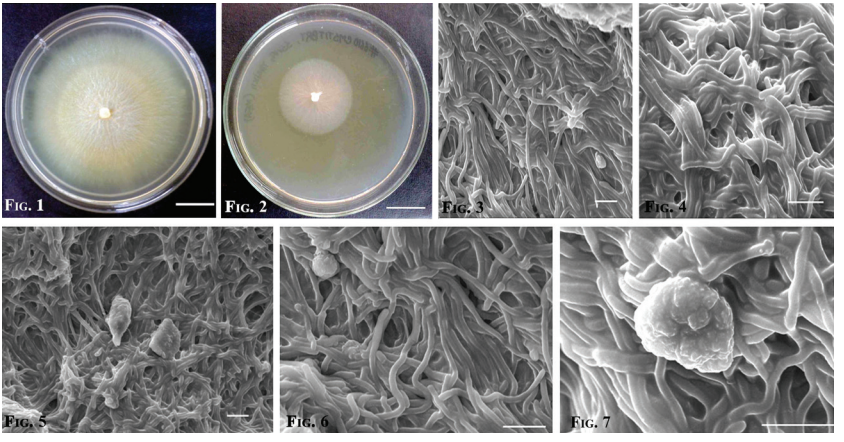
Differs from *Muscodor roseus* by its whitish to pale yellowish-orange mycelium and the absence of hyphal coils.

Type: India, Karnataka, BRT Wildlife Sanctuary, 11°43′–12°09′N 77°01–15′E as endophytic fungi from internal tissue of stem of *Cinnamomum zeylanicum* Blume (*Lauraceae*), 12 July 2009, leg. Sanjai Saxena #6610 (**Holotype,** NFCCI-2907; Gen Bank, JQ409999).

Etymology: The epithet *strobelii* honors Dr. Gary Strobel, Emeritus Professor, Department of Plant Sciences, Montana State University, Bozeman MT, USA.

Teleomorph: Unknown

In nature, the fungus is associated with *Cinnamomum zeylanicum* and is an ascomycete with sterile mycelia. The fungal colonies grew slowly over PDA and MEA media, with colony diameter of 17–18 mm on PDA and 27–29 mm on MEA when incubated at 24 ± 2 °C for 7 days with 12 hours of photoperiod. Fungal colonies on PDA are initially floccose and whitish but with age turn pale yellowish-orange. Over PDA it generates hyaline, septate branched hyphae that are 0.76–1.5 µm thick. Hyphae form fused cables that interweave into fishnet-like structures, which further merge to form zinnia flower- or bud-like



Figs. 1–7. *Muscodor strobelii* (#6610). 1. 30-day old colony on PDA. 2. Ropy appearance of mycelium after 6-day incubation on malt extract agar. 3. Mycelial arrangement of 10-day old culture on PDA (SEM). 4.Fishnet-like structures (SEM). 5–6. Zinnia flower- and bud-like structures after 10 days on PDA (SEM). 7. Enlarged view of zinnia flower-like structure (SEM). Bars: 1–2 = 2 cm; 3–7 = 10 μm.

structures (5.5–15.8 μm). The hyphal fabrication is denser in MEA, where the isolate produces interwoven mycelia that form a 0.76–2.04 μm diam. rope-like structure. The isolate did not produce any fruiting or conidial structure under tested in vitro conditions.

This *M. strobelii* isolate was found viable against the VOCs produced by *M. albus* CZ620 in the VOC stress bioassay on repeated exposure.

Detailed morphological studies using scanning electron microscopy

The mycelium of *M. strobelii* in SEM studies exhibits typical characters of a *Muscodor* species in forming rope-like strands that branch at a specific angle (Fig. 3). The cable-like mycelium further forms an interwoven ropy structure much like fishing net (Fig. 4) that culminates to form a unique structures at its terminal end to resemble a common zinnia flower- (as seen from the top) or bud-like (as seen from the side) structure (Figs. 5–7).

As these structures do not germinate further or sporulate, they should not be considered to be fruiting bodies. Morphologically, *M. strobelii* differs from *M. crispans* and *M. cinnamomi*, which exhibit cauliflower-like sterile structures and possess ropy coiled mycelia. *Muscodor yucatanensis* has a ropy structure with swollen hyphae while *M. albus* exhibits only a ropy mycelium, but both lack the sterile structures produced by *M. strobelii*. *Muscodor roseus,* with its dense rose coloured mycelium with a variable hyphal thickness that forms unique hyphal coiled structures, is remarkably dissimilar from *M. strobelii*.

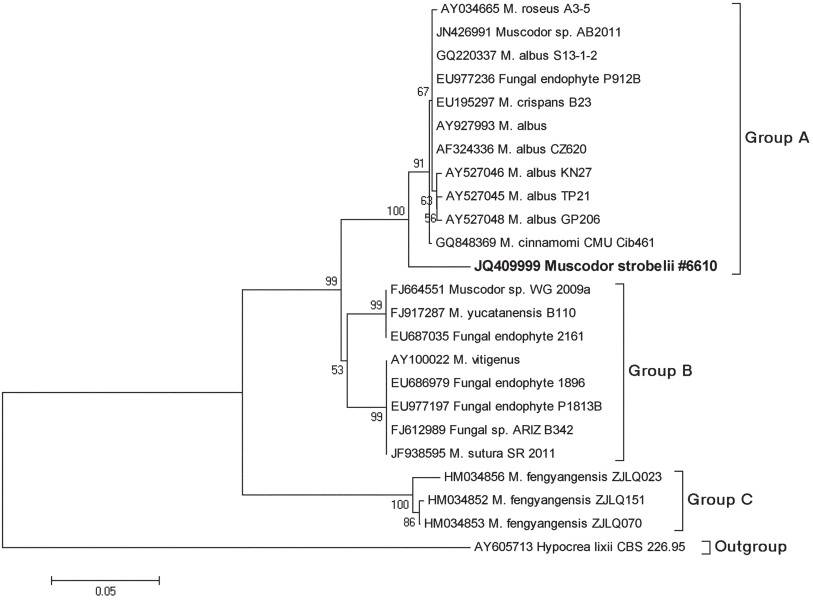


Fig. 8. Phylogeny of *Muscodor* spp.: Neighbor Joining tree based on ITS rDNA region. The optimal tree with the sum of branch length = 0.59173282 is shown. There were a total of 632 positions in the final dataset.

Molecular phylogeny of *M. strobelii*

The advanced BLAST search of the partial ITS1-5.8S-ITS2 rDNA region of *Muscodor strobelii,* which revealed a 94% sequence similarity with *M. cinnamomi* (GQ848369; Suwannarach et al. 2010), *M. crispans* B23 (EU195297; Mitchell et al. 2008), *M. albus* CZ620 (Worapong et al. 2001), *M. albus* E6, and *M. albus* GP206 and a 92% sequence similarity with *M. yucatanensis*, indicates that the isolate belongs to *Muscodor* genus (Table 1).

Representative ITS accessions of *Muscodor* species and unknown fungal endophytes (Table 1) were analyzed to establish possible phylogenetic relationships with *M. strobelii* through an alignment matrix using Clustal W, MEGA 5.0.5 analysis. The resultant phylogenetic tree divided into 3 major groups (Group A, B and Group C). Group A clustered *M. crispans, M. roseus*, *M. cinnamomi* CMU-Cib46*, M. albus* CZ620, 6 strains of *M. albus*, and one unknown fungal endophyte 912B with 91% bootstrap support; our isolate was found to be basal to group A with 100% bootstrap support. Group B comprised *M. yucatanensis, M. vitigenus, M. sutura, Muscodor* sp. WG2009a, and 4 unknown fungal endophytes. *Muscodor fengyangensis* species forms a separate group (Group C) with 100% bootstrap support; *Hypocrea lixii* CBS 226.95 was

Table 1. Advanced ITS-5.8 rDNA gene BLAST search homology analysis of *Muscodor strobelii*.

Species / voucher	GenBank no.	Query coverage	Sequence similarity
M. cinnamomi CMU Cib-461	GQ848369	98%	94%
M. crispans B23	EU195297	98%	94%
M. albus CZ620	AF324336	98%	94%
M. roseus A3-5	AY034665	98%	94%
M. sutura CA22-D	JF938595	91%	91%
M. vitigenus	AY100022	91%	91%
M. yucatanensis	FJ917287	90%	92%
M. fengyangensis ZJLQ070	HM034853	90%	90%
M. albus S13-1-2	GQ220337	98%	94%
M. albus I-41.3s	AY927993	98%	94%
M. albus TP21	AY527045	98%	94%
M. albus KN27	AY527046	98%	94%
M. albus GP206	AY527048	98%	94%
Muscodor sp. WG2009a	FJ664561	90%	92%
Fungal endophyte 912B	EU977236	98%	94%
Fungal endophyte 2161	EU687035	90%	92%
Fungal endophyte 1896	EU686979	91%	100%
Fungal endophyte P1813B	EU977197	91%	91%
Fungal sp. ARIZ B342	FJ612989	91%	91%

included as outgroup (Fig. 8). These data delineate *Muscodor strobelii* from the already described *Muscodor* species and highlight its novelty.

Bioassay of *Muscodor* species VOCs against human and plant pathogens

The volatiles emanated by *M. strobelii* #6610 exhibited a broad spectrum of activity against yeasts, bacteria, and filamentous fungi. Complete inhibition was observed in clinical isolates of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* after three days of exposure. The VOCs were bactericidal, as the test bacteria had lost their viability when they were re-inoculated on fresh medium. The test *Candida* isolates also showed complete inhibition when exposed to the *M. strobelii* VOC mixture and lost their viability.

Among the filamentous fungi, only *Penicillium citreonigrum*, *Botrytis cinerea*, and *Aspergillus japonicus* were completely inhibited by the VOC followed by *Cercospora beticola* and *Mycosphaerella fijiensis,* inhibited by 60–70%. The completely inhibited fungi were non-viable, whereas *Cercospora beticola*, *Rhizoctonia solani,* and *Colletotrichum gloeosporioides* showed delayed

Table 2. Antimicrobial activity of volatile organic compounds (VOCs) produced by *Muscodor strobelii* after 3 days exposure.

Test Cultures	Repository	% Growth (3 days exposure)
Bacillus subtilis	MTCC 441	70.3 ± 1.5
Escherichia coli	GMCP	0
Pseudomonas aeruginosa	GMCP	0
Staphylococcus aureus	GMCP	0
Candida albicans	JNU	0
Candida albicans	MTCC 3019	0
Candida albicans	MTCC 183	0
Penicillium citreonigrum	MTCC 160	0
Botrytis cinerea	MTCC 359	0
Aspergillus japonicus	MTCC 1975	0
Mycosphaerella fijiensis	MSU	43 ± 2.64
Cercospora beticola	MSU	32 ± 2
Rhizoctonia solani	MTCC 4634	60.7 ± 1.15
Colletotrichum gloeosporioides	MTCC 9623	63 ± 2
Fusarium oxysporum	DBTES, TU	70 ± 4
Lasiodiplodia theobromae	DBTES, TU	60 ± 4

*The growth pattern was compared with that of the control plate where the test bacteria, yeast, and filamentous fungi grew under no stress conditions. All tests were performed in triplicate; their mean ± SD was calculated.

Repositories:
DBTES, TU: Department of Biotechnology and Environmental Sciences, Thapar University, Patiala.
JNU: Jawaharlal Nehru University, New Delhi.
GMCP: Government Medical College, Patiala.
MSU: Montana State University, USA.

sporulation compared to the control (Table 2). The *M. strobelii* VOCs are active on fungi as well as bacteria. *Muscodor albus, M. crispans,* and *M. fengyangensis* also exhibit antibacterial as well as antifungal activity; however differential activity exists among the tested bacterial and fungal isolates, which might be attributed to the chemistry and quantity of the volatiles being produced.

Volatile compounds of *Muscodor strobelii*

A 10-day old culture of *M. strobelii* #6610 produced at least 14 volatile moieties that could be possibly identified based on GC/MS comparison of authentic standards obtained from commercial sources as well as by organic synthesis. Primarily the compounds were identified based on their mass spectral properties when compared to the NIST database. Of all the compounds produced, 4-octadecylmorpholine was the most abundant, with the highest peak area (12.5%). The other unique volatile compounds produced were Tetraoxapropellan, Aspidofractinine-3-methanol, 1-(3,5-di-tert-butyl-

Table 3. Volatile compounds[1] produced by *Muscodor strobelii*.

Retention time		Possible name	Molecular Formula	Mass (Da)
3.03	1.76	1-methyl-4-(prop-1-en-2-yl)cyclohex-1-ene	$C_{10}H_{16}$	136.23
3.69	4.56	Terpinolene	$C_{10}H_{16}$	136.23
13.13	3.5	2-morpholinoethanamine	$C_6H_{14}N_2O$	130.18
15.06	8.57	1-(3,5-di-tert-butyl-4-hydroxyphenyl)propan-1-on	$C_{17}H_{26}O_2$	262.38
15.33	9.35	Tetraoxapropellan	$C_{10}H_{16}O_4$	200.24
15.92	8.07	Viridiflorol	$C_{15}H_{26}O$	222
16.30	4.11	3,5-di-tert-butylcyclohexa-3,5-diene-1,2-dione	$C_{14}H_{20}O_2$	220.30
16.94	8.04	2-(6-tert-butyl-1,1-dimethyl-2,3-dihydro-1H-inden-4-yl)acetic acid	$C_{17}H_{24}O_2$	260
17.22	2.96	4-(2-morpholinoethyl)-4H-1,2,4-triazole-3-carboxamide	$C_9H_{15}N_5O_2$	225.25
20.193	1.08	1,2,3,4-tetramethyl-5,6-dineopentylbenzene	$C_{20}H_{34}$	274.48
21.178	12.53	4-octadecylmorpholine	$C_{22}H_{45}NO$	339.6
24.798	3.62	octadec-9-enoic acid	$C_{18}H_{34}O_2$	282.5
32.054	9.23	Aspidofractinine-3-methanol	$C_{20}H_{26}N_2O$	310.4
32.385	2.15	(2E)-2-(3-methoxy-5-methyl benzylidene)-7-methyl-1-indanone	$C_{19}H_{18}O_2$	278

[1] After 10 days incubation at 24 ± 2 °C on potato dextrose agar (PDA) using solid-phase micro-extraction (SPME) fibre and GC/MS analysis

4-hydroxyphenyl)propan-1-one, viridiflorol, terpinolene, 2-(6-tert-butyl-1,1-dimethyl-2,3-dihydro-1H-inden-4-yl)acetic acid, 3,5-di-tert-butylcyclohexa-3,5-diene-1,2-dione, and 2-morpholinoethanamine (Table 3). The volatiles commonly produced by other *Muscodor* species consist of propanoic acid, 2-methylesters, azulene and naphthalene derivatives, and thujopsene. *Muscodor strobelii* produces volatiles belonging to ketones, amines, carboxylic acids, and alcohols; they are unique and have not been reported from any other *Muscodor* species.

Conclusions

As *Muscodor* species are sterile and do not produce any reproductive structures, critical examination of cultural, physiological, and biochemical properties along with molecular phylogenetic data is essential for verifying them as distinct species. *Muscodor strobelii* exhibited a variety of common features shared by *Muscodor* species; it possessed, however, some unique features like differences at the molecular level, unique morphological features seen through scanning electron microscopy, differing antimicrobial susceptibility data, and a unique volatile gas mixture composition (Table 4). Thus *M. strobelii* is introduced as a novel species that possesses potential as a mycofumigant.

Table 4. Comparison of *M. strobelii* with other *Muscodor* species.

	strobelii	*albus**	*cinnamomi**	*crispans**	*fengyangensis**	*roseus**	*sutura**	*vitigenus**	*yucatanensis**
Mycelial Growth	Rope-like, slimy; Zinnia- & bud-like bodies	Rope-like	Rope-like with coiled hyphae	Rope-like with cauli-flower-like bodies	Rope-like with coiled hyphae	Rope-like, forming erumpent pie-shaped sectors	Rope-like bands extra-cellular bodies	Rope-like	Rope-like with coiled hyphae
Hyphal growth (at colony front)	Straight	Straight	Straight	Heavily wavy new growth	Straight	Intertwining, rope-like, (occasionally coiled)	Suture-like pattern on PDA (new growth)	Straight	Multiple rope-like strands (can inter-twine to form coils)
Pigment production	Pale yellow (in light)	None	Pale orange (in light)	Reddish (in light)	Yellow	Light rose	Reddish (in dark)	None	None
Major VOCs	4-octadecyl-morpholine, Tetraoxa-propellan, Aspido-fractinine-3-methanol	Propanoic acid, 2-methyl, 2-nonanone, Naphthalene & azulene derivatives	Propanoic acid, 2-methyl, Methyl ester, β-humulene	Propanoic acid, 2-methyl, (+ many esters)	Naphthalene & azulene derivatives (+ many others)	2-butenoic acid, Ethyl ester, 1,2,4-tri-methyl-benzene, 2-nonadiene	Propanoic acid, 2-methyl, Thujopsene	Naphthalene (only)	1R,4S,7S, 11R-2,2,4,8-Tetramethyl-tricyclo [5.3.1.0(4,11)] undec-8-ene, Caryophyl-lene, Aroma-dendrene
Bioactivity									
Antifungal	+	+	+	+	+	+	+	–	+
Antibacterial	+	+	+	+	+	+	–	–	–
Anti-insect	–	–	–	–	–	–	–	+	–

* Data collated from the protologue publications

Acknowledgements

The authors thank the National Biodiversity Development Board, Department of Biotechnology (NBDB-DBT), New Delhi, India for providing financial support under project no. BT/PR/10083/NDB/52/95/2007. They express their gratitude to Prof. Dr. Gary A. Strobel (Professor Emeritus, Montana State University, Bozeman MT, USA) for providing the *M. albus* CZ620 type strain, fungal pathogens, and valuable suggestions. We appreciate the kind help of Dr. Ruchita Pal and Mr. Ajay Kumar (Advanced Instrumentation and Research Facilities (AIRF), JNU, New Delhi) for SEM and GC/MS analysis. We are also grateful to Prof. Strobel, Dr. Sanjay K Singh (Agharkar Research Institute, Pune, Maharashtra, India), and Dr. G.S. Dhingra (Punjabi University, Patiala, Punjab, India) for presubmission reviews.

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